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APPLICATION FOR UNITED STATES LETTERS PATENT IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

(Case No. 01-660)

Title: **FLUORESCENCE POLARIZATION-BASED HOMOGENEOUS ASSAY FOR AFLATOXINS**

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BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates to the field of mycotoxin assays. More particularly, this invention relates to a homogeneous assay that uses changes in fluorescence polarization to detect the presence of aflatoxins in agricultural products.

2. Description of Related Art

Aflatoxins are mycotoxins produced by *Aspergillus flavus* molds[1]. Aflatoxins have been known for a long time, but their carcinogenicity was first detected in the late 1960s[4]. Various forms of aflatoxin, including B_1, B_2, G_1, and G_2 and many others, have been found in many forms of human foods, such as cereals, grains and peanut products[9,11]. Aflatoxin B_1 is the most toxic and most abundant of all. An exposure to aflatoxins has been associated with an increased incidence of primary hepatocellular carcinoma[7].

Due to their toxicity and carcinogenicity, various analytical methods have been devised to quantitatively determine the amount of aflatoxin in agricultural products[1-4,6,8]. One difficulty with such assays is that aflatoxins are very hydrophobic and therefore very insoluble in aqueous solvents. Thus, mixtures of organic solvent with water have generally been used to extract aflatoxins from samples.

Another difficulty is that most of the common assays, including TLC and HPLC[10], require extended cleanup steps and derivatization after extraction in order to get rid of interfering substances. ELISA methods are relatively faster but are hard to

quantify due to various washing steps, liquid transfers and incubation times and cleaning steps.

Accordingly, there is a need for an assay for the determination of aflatoxins in agricultural products that is rapid, simple to apply, and that can yield quantitative results.

SUMMARY OF THE INVENTION

In a first principal aspect, the present invention provides a homogeneous assay for the determination of aflatoxins in agricultural products. Aflatoxin is extracted from a sample, and the extract is combined with a tracer and an antibody to provide a mixture. The antibody is specific for aflatoxin. The tracer comprises an aflatoxin oxime conjugated to a fluorophore. The tracer is able to bind to the antibody to produce a detectable change in fluorescence polarization. The fluorescence polarization of the mixture is measured and compared to a standard curve.

In a second principle aspect, the present invention provides an assay kit for the determination of aflatoxins in agricultural products. The assay kit comprises an antibody and a tracer, each in an amount suitable for at least one assay, and suitable packaging. The antibody is specific for aflatoxin. The tracer comprises an aflatoxin oxime conjugated to a fluorophore. The tracer is able to bind to the antibody to produce a detectable change in fluorescence polarization.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph showing the change in fluorescence polarization over time for a range of aflatoxin concentrations, in accordance with an embodiment of the present invention.

Figure 2 is a graph showing the change in fluorescence polarization over time for a sample containing no aflatoxin and a range of methanol concentrations, using the data of Table 1, in accordance with an embodiment of the present invention.

Figure 3 is a standard curve for a fluorescence polarization assay for aflatoxins, using the data of Table 2, in accordance with an embodiment of the present invention.

Figure 4 is a graph comparing the aflatoxin concentration of samples as measured using HPLC with the aflatoxin concentration as calculated from the standard curve of Figure 3, in accordance with an embodiment of the present invention.

Figure 5 is graph comparing the aflatoxin concentration of spiked samples with the aflatoxin concentration calculated from measurements of fluorescence polarization, in accordance with an embodiment of the present invention.

Figure 6 is a standard curve for a fluorescence polarization assay for aflatoxins, used to obtain the data in Tables 6 and 7, in accordance with an embodiment of the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The preferred embodiments of the present invention provide a relatively simple homogeneous assay for the determination of aflatoxins in agricultural products that is based on measurements of fluorescence polarization. The technique of fluorescence

polarization has been successfully utilized in various assay involving proteins, enzymes, drugs, DNA, hormones, peptides and antibodies.

The principle behind the fluorescence polarization technique is as follows. Fluorescent probes having low molecular weight have low polarization values due to their fast rotation, whereas fluorescent probes with higher molecular weight have higher polarization due to their slower rotation. Thus the polarization value of a fluorophore increases upon binding to a larger molecule. Further information about the fluorescence polarization technique is provided in U.S. Patent Nos. 5,427,960 and 5,976,820 and in Nasir, M. S. and Jolley, M. E., "Fluorescence Polarization: An analytical tool for Immunoassay and Drug Discovery," *Combinatorial Chemistry & High Throughput Screening*, **1999**, *2*, 177-190, which references are incorporated herein by reference.

In the present invention, aflatoxin extracted from a sample competes with a fluorescent tracer in the presence of a monoclonal antibody, thereby giving rise to a change in fluorescent polarization that is dependent upon the aflatoxin concentration.

The preferred embodiments of the present invention provide a homogeneous assay for aflatoxin that is sensitive, rapid, simple, and inexpensive. It can also be field-portable and yield quantitative results.

1. Materials and Methods

Two different aflatoxin monoclonal antibodies were used in these studies. An aflatoxin monoclonal antibody purchased from Sigma (catalog no. A-9555) was used in initial assay development work, but it was found to have sensitivity to methanol. In later work, a monoclonal antibody, available from Dr. Chris Maragos of the Agricultural

Research Unit of the United States Department of Agriculture (Peoria, Illinois), was used because it was found to be stable in methanol. Use of this monoclonal antibody was reported in Chris M. Maragos and Vicki S. Thompson, "Fiber-optic Immunosensor for Mycotoxins," *Natural Toxins* 7:371-376 (1999), which is incorporated herein by reference. In addition, many other monoclonal antibodies for aflatoxins are known. *See, e.g.,* U.S. Patent No. 4,835,100.

Samples of corn that were naturally contaminated with aflatoxins, and samples of aflatoxin-free popcorn were purchased from Trilogy Analytical Laboratory, Inc. (Washington, Missouri). Trilogy also provided an Aflatoxin $B_1/B_2/G_1/G_2$ (7/1/3/1) mixture. Pure Aflatoxin B_1 was obtained from Sigma.

Fluorescence polarization measurements were done at room temperature using a single tube Sentry-FP fluorescence polarization instrument (Diachemix Corp.).

2. Preparation of Aflatoxin Tracer

In a 10 ml round bottomed flask fitted with a magnetic stirrer and a condenser, Aflatoxin B1 (5 mg, 0.016 mmol, Sigma) and O-carboxymethyl-hydroxylamine-hemihydrochloride (41 mg, 0.19 mmol, Sigma) were mixed with 1.2 ml absolute ethanol. To this solution, 230 µl of a 2 M NaOH solution (0.46 mmol) was added with stirring, and the solution refluxed for 3 hours. The resultant solution was stirred overnight at room temperature, concentrated on a rotary evaporator, and diluted to 1.5 ml with water. Drops of 1 N NaOH were added to adjust the pH to ~9, and the solution was washed with ethyl acetate (using two portions of about 3 ml each). The aqueous layer was acidified with 6 M HCl to a pH ~2, and the resultant mixture was stored at 0° C in a refrigerator.

Some solid precipitated, which was separated and dried. TLC on silica using ethyl acetate:MeOH:NH_4OH (32:17:5) gave a major spot at Rf ~0.5 corresponding to the oxime product.

A 20 µl THF solution of (Aflatoxin B_1)-O-carboxymethyloxime, prepared as described above, was mixed with 20 µl of dicyclohexylcarbodiimide (DCC) in methylene chloride (10 mg/ml) and 200 µl of methylene chloride. After 2-3 minutes, a 20 µl THF solution of fluoresceinamine (isomer 2, 10 mg/ml) was added. The reaction was performed overnight at room temperature. As a control, the same reaction was also run without the aflatoxin oxime. TLC of the products (using $CHCl_3$:MeOH:CH_3CO_2H, 40:10:3) showed many spots. One spot at Rf ~0.7 was found to be absent in the TLC run of the control. This tracer was collected, dissolved in MeOH, and then diluted in buffer so as to give an intensity of ~400,000 relative fluorescence units when 10 µl of this tracer solution was added to 1 ml of buffer.

It was observed that this tracer gave a stable polarization of ~40 mP. After adding 10 µl of 1/50 diluted antibody (Sigma, A-9555) to give a final dilution of 1/5,000, the polarization slowly increased to ~230 mP in a period of five minutes.

In order to confirm the reactivity, 1 ml buffer was taken and mixed with 10 µl of antibody and 10 µl of (0.8 mg/ml) Aflatoxin B_1. The mixture was kept at room temperature for five minutes and then blanked in the FP instrument. After 10 µl of tracer was added, the fluorescence polarization decreased from ~230 mP to ~41 mP.

A different Aflatoxin B_1 tracer was prepared using a similar reaction but with fluoresceinamine (isomer 1) as the fluorophore. The resulting tracer showed less sensitivity than when fluoresceinamine (isomer 2) was used as the fluorophore.

Specifically, the starting polarization (~32mP) changed to only ~140 mP when the antibody was added.

Aflatoxin B_1 tracers were also prepared using other amine derivatives of fluorescein as the fluorophore. The results are summarized as follows. When 5-aminoacetyl-amidofluorescein (5AAF) was used, the resulting tracer had a fluorescence polarization of ~30 mP, which increased to ~65 mP when antibody was added. When 5-(5-aminopentyl)-thioureidyl fluorescein (5,5APTF) was tried as the fluorophore, the tracer had a fluorescence polarization of ~35 mP, which increased to ~102 mP upon antibody addition. Fluorescein thiosemicarbazide (FTSC), 4-aminomethyl fluorescein, and 5-aminomethyl fluorescein were also tried, but these fluorophores did not result in any active product, i.e., they showed no significant change in fluorescence polarization upon binding to antibody. Therefore, it was concluded from these studies that isomer 2 of fluoresceinamine gave the best results for this antibody.

3. Assay Development

Aflatoxin antibody was purchased from Sigma (A-9555) and used for initial assay development after diluting it (1/150,000) in PBSA-BGG (pH ~7.5), which is a phosphate buffer solution containing 1 gram per liter sodium azide and 9 grams per liter sodium chloride, with bovine gamma globulin (BGG) present at a concentration of 100μg/ml. 1 ml of the antibody solution was mixed with 50 μl of a solution having a known concentration of free Aflatoxin B_1 in methanol/water (70/30). Aflatoxin B_1 concentrations varying from 0 to 40 ppb were used. After taking a blank measurement, 10 μl of diluted tracer, prepared as described above using fluoresceinamine (isomer 2) as

the fluorophore, was added, and the change in fluorescence polarization was monitored for 10 minutes. The results are shown in Figure 1.

Since aflatoxins are typically extracted from agricultural samples using a mixture of an organic solvent and water, the effect of methanol on the Aflatoxin antibody from Sigma (A-9555) was studied. Specifically, 1 ml of diluted antibody solution was mixed with a known concentration of methanol. After measuring a blank, tracer was added (the Aflatoxin B_1 oxime-isomer 2 fluoresceinamine tracer described above), and the fluorescence polarization was measured over time. The results are summarized in Table 1 below and in Figure 2.

TABLE 1

Time (Minutes)	50ml MeOH	40ml MeOH	30ml MeOH	20ml MeOH	10ml MeOH	50ml water
0.5	32.0	49.70	88.80	56.70	73.20	46.30
1.0	91.0	105.40	74.40	117.60	128.40	129.80
1.5	117.0	126.20	126.30	143.00	156.70	165.40
2.0	145.9	140.50	139.30	157.70	170.40	178.60
2.5	150.4	152.50	154.30	168.90	179.20	188.30
3.0	161.3	161.50	167.50	176.70	186.80	194.30
3.5	165.5	165.70	170.30	181.90	192.10	199.60
4.0	168.1	175.40	178.60	186.10	195.50	202.30
4.5	179.6	177.10	185.10	191.30	198.70	205.40
5.0	189.3	179.10	186.80	195.50	202.10	208.30
5.5	186.1	185.80	187.10	198.40	204.50	209.80
6.0	186.6	187.00	190.40	198.00	205.60	210.60

These results show that the fluorescence polarization decreases as the methanol concentration increases, and, for a given methanol concentration, the fluorescence polarization increases over time. Thus, although the tracer itself is stable in methanol and related organic solvents for an extended period of time, these results suggest that this

antibody is sensitive to methanol. As a result, while this antibody has the sensitivity for use in a fluorescence polarization-based assay for aflatoxins, it is preferable to use an antibody that is more stable in methanol in order to yield more reliable results. It was found that the monoclonal antibody available from Dr. Chris Maragos of the Agricultural Research Unit of the United States Department of Agriculture (Peoria, Illinois) had the desired stability in methanol.

4. FP Assay for Aflatoxins in Naturally Contaminated Corn Samples

Corn samples that were naturally contaminated with aflatoxins were purchased from Trilogy Analytical Laboratory, Inc. (Washington, Missouri). Aflatoxin was extracted from each 20 g sample of crushed grain using 100 ml mixture of MeOH/water (70/30) in duplicate by shaking each sample from time to time for about 30 minutes. Extracts were filtered through a fine filter paper and stored in sealed bottles at room temperature for analysis.

Standards were prepared in MeOH/water (70/30) by diluting a concentrate of Aflatoxin $B_1/B_2/G_1/G_2$ (7/1/3/1) provided by Trilogy into various concentrations. 40 μl of each sample or standard was mixed into 1 ml antibody solution (1/150,000 in PBSA-BGG buffer) in a test tube. The antibody used was the methanol resistant antibody provided by Dr. Chris Maragos. After blanking each sample, 10 μl of tracer was added into each tube, the samples were incubated for 15 minutes at room temperature, and then the fluorescence polarization was measured for each tube. The tracer that was used was the Aflatoxin B_1 oxime-isomer 2 fluoresceinamine tracer described above. A standard

curve was plotted using duplicate values. The fluorescence polarization values for the standards are shown below in Table 2 and in Figure 3.

TABLE 2

Aflatoxin Concentration (ppb)	mP (first run)	mP (second run)
0.0	153.0	160.0
10.0	134.0	135.0
20.0	128.0	130.0
40.0	97.0	96.0
60.0	78.0	79.0
80.0	71.0	72.0
100.0	67.0	67.0
160.0	64.0	60.0

The concentration of aflatoxin in each corn sample was then calculated from the standard curve. The results are summarized in Table 3 below.

TABLE 3

Sample	mP (first run)	mP (second run)	Calculated aflatoxin concentration (ppb)
1	163.0	159.0	0
2	143.5	135.0	8.99
3	80.0	75.5	69
4	92.0	90.5	47
5	98.0	112.5	32.61
9	73.5	76.5	75.16
7	75.5	71.0	79.94
8	83.0	87.0	56
10	125.0	124.5	17.35
11	146.5	146.0	5.4

These samples were also analyzed using both the fluorescence polarization protocol described above and by standard HPLC techniques, and the aflatoxin

concentrations determined using these two techniques were compared. The results are summarized below in Table 4 and in Figure 4, except for the results from one sample that had a very high level of aflatoxin contamination. These results show a good correlation between HPLC and FP ($r^2 = 0.97$).

TABLE 4

Aflatoxin concentration from HPLC (ppb)	**Aflatoxin concentration from FP (ppb)**
0.50	0.0
10.20	9.0
56.40	69.0
41.50	47.0
33.30	32.6
47.10	56.0
60.80	75.0
75.00	80.0
13.01	5.4

5. Analysis Aflatoxin in Popcorn Samples

20 g of crushed samples of aflatoxin-free popcorn were spiked with an Aflatoxin $B_1/B_2/G_1/G_2$ mixture (7/1/3/1) to a known concentration. The fluorescence polarization analysis was performed in duplicate on each extract as reported above. The aflatoxin concentration of each sample was calculated from the average measured fluorescence polarization, using a calibration curve, and compared with the known spiked concentration. The results are summarized in Table 5 below and in Figure 5. In this study, the sample spiked to 320 ppb was diluted 1/10 for the fluorescence polarization measurement.

TABLE 5

Spiked Aflatoxin Concentration (ppb)	mP	Calculated Aflatoxin Concentration (ppb)
0	178	0.47
10	169	5.79
10	168	6.43
20	157	14.60
20	159	13.16
30	148	23.30
40	142	31.12
80	129	57.72
160	122	94.74
320	152	189

These results show a good correlation between theoretical values and the results obtained using the fluorescence polarization assay of the present invention ($r^2 = 0.996$). However, the fluorescence polarization results consistently underestimated the actual aflatoxin concentration. One explanation is that naturally contaminated samples had mainly B_1 and some B_2, but no G_1 and G_2, whereas the popcorn samples were spiked with a mixture of $B_1/B_2/G_1/G_2$ in a 7/1/3/1 ratio.

To test this explanation, the cross-reactivity of these aflatoxins was investigated. Aflatoxin B_1, B_2, G_1 and G_2 were purchased from Sigma and individually diluted in a mixture of methanol/water (70/30) to give a range of concentrations, namely, 10, 25, 40, 80, and 100 ppb. The standard curve shown in Figure 6 was obtained by performing the fluorescence polarization assay described herein on the Aflatoxin B_1 solutions. Table 6 and Table 7 show the results for Aflatoxin G_1 and G_2, respectively, calculating the aflatoxin concentrations from the calibration curve of Figure 6.

TABLE 6

Aflatoxin G_1 Concentration (ppb)	mP	Calculated Aflatoxin Concentration (ppb)
10	143	3.75
25	130	9.06
40	115	16.44
80	101	25.16
100	99	26.61

TABLE 7

Aflatoxin G_2 Concentration (ppb)	mP	Calculated Aflatoxin Concentration (ppb)
10	144	3.37
25	133	7.76
40	113	17.56
80	101	25.16
100	98	27.35

These results show that the concentrations of Aflatoxin G_1 and G_2 are underestimated when they are calculated from a calibration curve obtained from Aflatoxin B_1 alone. More particularly, Aflatoxin G_1 and G_2 both cross-react with Aflatoxin B_1 only to the extent of 30-40%. This may explain the underestimation of aflatoxin concentration observed in the spiked popcorn samples.

6. Assay Kit

The materials used to perform the assay of the present invention are preferably made available in kit form. The kit preferably includes a quantity of extraction solution for extracting aflatoxin from samples of grain or other products, tracer and antibody in an amount suitable for at least one assay, along with suitable packaging and instructions for use. The tracer and antibody may be provided in

solution, as a liquid dispersion, or as a substantially dry powder (e.g., in lyophilized form).

The suitable packaging can be any solid matrix or material, such as glass, plastic, paper, foil, and the like, capable of separately holding within fixed limits the buffer, tracer, and antibody. For example, the extraction solvent, tracer, and monoclonal antibody may be provided as solutions in separate labeled bottles or vials made of glass or plastic.

The antibody is specific for aflatoxins and is preferably a monoclonal antibody. Most preferably, the monoclonal antibody is stable in the extraction solvent.

The tracer comprises a fluorophore conjugated to an aflatoxin oxime, preferably (Aflatoxin B_1)-O-carboxymethyloxime. Suitable fluorophores include fluoresceinamine (isomer 1), fluoresceinamine (isomer 2), 5-aminoacetyl-amidofluorescein (5AAF), 5-(5-aminopentyl)-thioureidyl fluorescein (5,5APTF). Other fluorophores may be used, provided the resulting tracer is able to bind with the antibody to produce a detectable change in fluorescence polarization. Preferably the fluorophore is fluoresceinamine. Most preferably, the fluorophore is fluoresceinamine (isomer 2).

The extraction solvent is preferably a mixture of an organic solvent, such as methanol or acetonitrile, in water. Most preferably, the extraction solvent is a methanol/water (70/30) mixture.

7. References

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